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Title: *Expression, purification and metal utilization of recombinant SodA from Borrelia burgdorferi*

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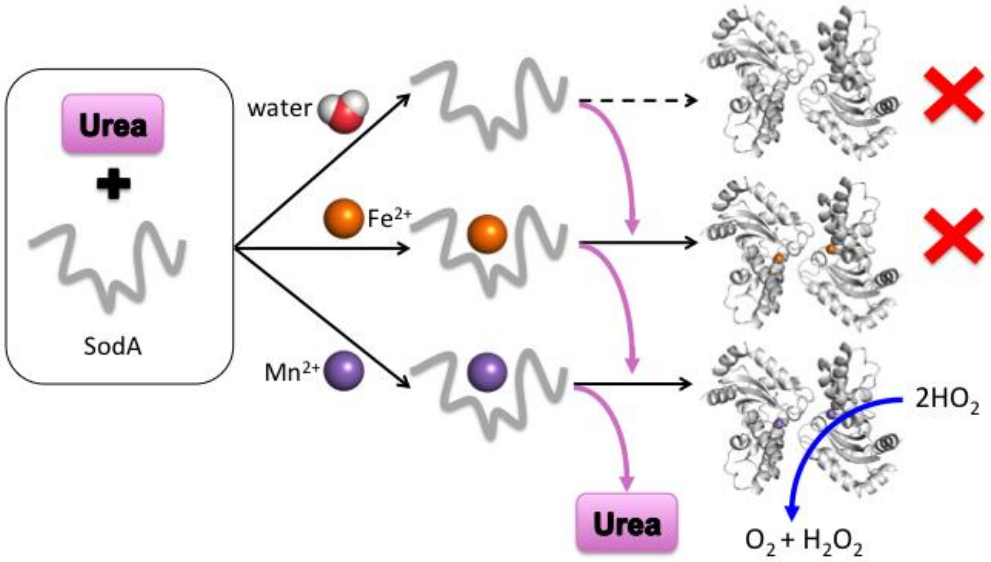
Abstract
Borrelia are microaerophilic spirochetes capable of causing multisystemic diseases such as Lyme disease and Relapsing Fever. The ubiquitous Fe/Mn-dependent superoxide dismutase (SOD) provides essential protection from oxidative damage by the superoxide anion. *Borrelia* possess a single SOD enzyme - SodA that is essential for virulence, providing protection against host-derived reactive oxygen species (ROS). Here we present a method for recombinant expression and purification of *Borrelia burgdorferi* SodA in *E. coli*. Metal exchange or insertion into the Fe/Mn-SOD is inhibited in the folded state. We therefore present a method whereby the recombinant *Borrelia* SodA binds to Mn under denaturing conditions and is subsequently refolded by a reduction in denaturant. SodA purified by metal affinity chromatography and size exclusion chromatography reveals a single band on SDS-PAGE. Protein folding is confirmed by circular dichroism. A coupled enzyme assay demonstrates SOD activity in the presence of Mn, but not Fe. The apparent molecular weight determined by size exclusion corresponds to a dimer of SodA; a homology model of dimeric SodA is presented revealing a surface Cys distal to the dimer interface. The method presented of acquiring a target metal under denaturing conditions may be applicable to the refolding of other metal-binding proteins.

Keywords: *Borrelia*; Superoxide dismutase; Manganese; *E. coli*

Graphical abstract



Highlights

- Recombinant *Borrelia burgdorferi* SodA is active in the presence of Mn, but not Fe
- *B. burgdorferi* SodA is a homodimer
- Recombinant *B. burgdorferi* SodA can be refolded from 8M urea in the presence of Mn

Acknowledgements

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1. Introduction

Superoxide ($O_2^{\bullet -}$), formed by the reduction of O_2, is a reactive free radical capable of generating numerous other free radicals and reactive oxygen species that may go on to damage cellular components [1]. Superoxide dismutase (SOD) is highly conserved throughout all domains of life and catalyzes the dismutation of $O_2^{\bullet -}$ to hydrogen peroxide (H_2O_2) [2]. The most ancient form, thought to have evolved over 2 Gyr ago, is the Fe/Mn-SOD [3]. Found in mitochondria (SOD2) and the cytoplasm of bacteria, the Fe/Mn-SOD exists as either a homodimer or homotetramer with one metal-binding site per chain [2].

Borrelia burgdorferi is a pathogenic spirochete with several characteristics that are dissimilar to other prokaryotes, including an unusual genome structure [4], extremely limited metabolic capacity [5], and the presence of polyunsaturated membrane lipids [6]. *Borrelia* is also highly unusual in its ability to grow without needing a source of Fe [7], instead its cellular metabolism has evolved to utilize Mn, which is imported to significant concentrations by the specific transporter BmtA [8]. No iron-specific enzymes have been identified in *Borrelia*, however Fe-binding proteins have been identified that play a role in Cu/Fe detoxification [9]. The lack of cellular Fe gives some protection against oxidative damage to DNA by Fenton chemistry. The major targets of host-derived reactive oxygen species are the polyunsaturated membrane lipids [6].

While a small number of SOD-enzymes have been identified that remain catalytically active with either Mn or Fe bound at the active site (cambialistic SODs), the majority have evolved to function only in the presence of their target metal ion and are named correspondingly as either Fe-SOD or Mn-SOD. Fe/Mn-SODs bind to either metal with the same trigonal-bipyramidal geometry, and have high sequence and structural homology [10]. Metal coordinating residues (His26, His81, Asp167 and His171 based on *E. coli* numbering) are identical between the Mn and Fe-SODS and mis-incorporation can occur. Metal selectivity is thought to be at least partly driven by bioavailability. Predicting metal specificity from protein sequence alone is challenging [11].

The genome of *Borrelia burgdorferi* contains a single SOD (*sodA*, bb0153), which was demonstrated to be essential for infectivity in a mouse model [12]. The inactivation of *sodA* resulted in a phenotype more vulnerable to challenge by activated macrophages and neutrophils, while *in vitro* growth was unaltered. Although early work suggested this might be an Fe-SOD [13], it was subsequently demonstrated to utilize Mn [14]. An elegant series of *in vivo* experiments demonstrated that *Borrelia* accumulates high levels of cellular Mn, and that this is required to activate the SodA enzyme. In contrast, *Borrelia* SodA expressed in the iron-rich environment of yeast mitochondria was inactive [14].

Previous studies of the metal utilization of *Borrelia* SodA have not used pure SodA, rather assays based on non-denaturing electrophoresis gels with *Borrelia* whole cell lysate [13, 15] or purified cell extracts [14]. In addition, no

studies to date have conclusively determined whether this enzyme is cambialistic, whereby it could use both manganese and iron as its co-factor. Therefore, the objective of this work was to produce and purify recombinant *Borrelia* SodA allowing *in vitro* investigation of enzyme activity in the presence of Mn and Fe. Here we report a method to produce sufficient quantity of SodA to allow structural and biochemical studies. We describe a protocol that utilizes addition of Mn and Fe salts to recombinant protein in the denatured state prior to refolding and purification, thereby preventing contamination of downstream processes with these reactive transition metal ions. A homology model of dimeric SodA is presented.

2. Material and Methods

2.1 Plasmid construction and gene expression

The *bb0153* gene was codon optimized for expression in *E. coli*, synthetically produced (Eurofins Genomics) and sub-cloned into pET-47b(+) (Novagen®). The resulting construct was transformed into T7 Express Competent *E. coli* (New England BioLabs). Transformed cells were grown in LB media at 37 °C in an orbital incubator and were induced at an OD_{600} of 0.6 by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich) to a working concentration of 1 mM. After 4-hours, induced cells were harvested by centrifugation and lysed on ice by pulsed sonication in 0.3 M NaCl, 50 mM Tris-HCl, pH 8.0.

2.2 Purification of inclusion bodies

The soluble fraction was removed by centrifugation (20,000 x g for 30 min at 4 °C), the insoluble material was resuspended in 0.3 M NaCl, 50 mM Tris-HCl, pH 7 and briefly pulse sonicated at an amplitude of 40% for a total length of 30 seconds. DNase1 was added at 1 µg/ml and the solution incubated at 4 °C for 4 hours. Following incubation, samples were centrifuged (20,000 x g for 30 min at 4 °C) and weighed prior to re-suspension in Wash Buffer 1 (Table 1) at 10 ml per gram of pellet. The suspension was briefly sonicated as previously described. Following this, the suspension was subjected to low speed centrifugation (~8000 x g) until a firm pellet was obtained. This process was repeated twice with inclusion Wash Buffer 1, twice with Wash Buffer 2, and twice with Wash Buffer 3 (Table 1). The inclusion bodies were then solubilized in Denaturing Buffer overnight at room temperature with shaking.

Wash Buffer 1	0.3 M NaCl, 50 mM Tris-HCl, 1 mM EDTA, 10 mM DTT, 5 % Triton X-100, pH 8.0
Wash Buffer 2	0.3 M NaCl, 50 mM Tris-HCl, 1 % Triton X-100, pH 8.0
Wash Buffer 3	0.3 M NaCl, 50 mM Tris-HCl, pH 8.0
Denaturing Buffer	8 M urea, 0.3 M NaCl, 50 mM Tris-HCl, pH 8.0

Table 1. Buffers for inclusion-body wash procedure.

2.3 Purification of metal-free protein

Divalent metal ions present in the re-suspended bacterial pellet were removed by utilizing unchelated nitrilotriacetic acid (NTA). A 5 ml Ni-NTA column (His-Trap HP, GE Healthcare) was stripped of Ni^{2+} by addition of 100 mM EDTA. After equilibration of the column with Denaturing Buffer (8 M urea, 0.3 M NaCl, 50 mM Tris-HCl, pH 8.0), the resuspended pellet (volume 20 ml) was passed through the column twice to bind any free metal ions at a flow rate of 0.5 ml/min using an AKTA Prime FPLC system.

2.4 Addition of metal co-factors

Following the removal of metal ions by NTA the pH of the denatured protein solution was decreased to pH 4.0 by slow addition of dilute HCl. Acidification of the solution was required to both increase the solubility and minimize the

oxidation of Fe^{2+}. $MnCl_2$ or $FeCl_2$ were added to samples of recombinant SodA to a final concentration of 1 mM. Recombinant protein samples were incubated at 4°C overnight before the pH was increased back to pH 8.0 by slow addition of dilute NaOH.

2.5 On-column refolding and purification

Recombinant protein samples (with addition of either $MnCl_2$ and $FeCl_2$ or control) were subjected to centrifugation at 16,000 g for 30 minutes prior to refolding/purification using a His-Trap HP column (GE Healthcare). The column was equilibrated with 10 column volumes (CV) of denaturing buffer (8 M urea, 50 mM Tris-HCl, 0.3 M NaCl, pH 8.0) and recombinant protein loaded at 0.5 ml/min. Protein folding was achieved by washing with a linear gradient from 8 M to 1 M urea over 2 hours in 50 mM Tris-HCl, 0.3 M NaCl, pH 8.0 (flow rate 0.5 ml/min). The column was then washed with 10 column volumes of 1 M urea, 50 mM Tris-HCl, 0.3 M NaCl, 50 mM imidazole, pH 8.0. Recombinant protein was eluted in 50 mM Tris-HCl, 0.3 M NaCl, 0.3 M imidazole at pH 8.0.

Size Exclusion Chromatography was carried out using a Superdex 75 10/300 GL column (GE Healthcare) in 50 mM Tris-HCl, 0.3 M NaCl at pH 8.0. The exclusion volume/void volume was determined using blue dextran (molecular mass of ~2,000 kDa) which eluted at 8.7 ml. Apparent molecular weights were determined by comparison with a calibration curve prepared using known protein standards (Sigma-Aldrich).

Mn-SodA, Fe-SodA and apo-SodA were concentrated using a 10NMWL Amicon Ultra-15 centrifugal filter unit (Merck Millipore), centrifuged at 16,000 x g for 20 min at room temperature and protein concentrations ascertained by UV spectroscopy (extinction coefficient 38390 $M^{-1}cm^{-1}$) and Bradford analysis [16].

2.6 Circular dichroism

Circular dichroism experiments were carried out on a Jasco J-810 spectropolarimeter. Air and buffer blank background data were acquired and experimental measurements taken over a wavelength range of 185- 260 nm. Experimental data was obtained at a protein concentration of 0.1 mg/ml over five individual scans and averaged. Data were analyzed using DichroWeb [17, 18] using a mean residue weight of 111.8 Da, corresponding to the mean residue weight of SodA. Algorithms used for deconvolution included CDSSTR, CONTIN and SELCON3 with the SP175 reference set.

2.7 SOD enzyme activity assay

Superoxide dismutase activity was measured by an indirect enzyme assay (SOD Assay Kit, 19160, Sigma-Aldrich) and carried out according to the manufacturer's instructions with additional negative controls to account for possible interference from the utilized buffers. The assay was used to compare activity rates of recombinant Mn-SOD, Fe-SOD and apo-SOD and was run in in quadruplicates with a protein concentration of 100 µg/ml in 96 well plates in a total well volume of 240 µl. Formazan dye production was determined by measuring absorbance for 20 minutes at 440 nm using a BMG Labtech SPECTROstar Nano microplate-reader. Statistical analysis by ANOVA and a multiple comparison of sample means by Tukey's test (TukeyHSD) was conducted in R [19].

2.8 Homology modelling

A homology model of *B. burgdorferi* SodA (UniProtKB accession code O30563) was generated using Phyre2 using intensive mode [20]. The highest ranked template was the Mn-dependent SOD from *Thermus thermophilus* with 50% sequence identity over 201 residues. The homodimer was generated by superimposition of the Phyre2 model onto the dimers of Mn-SOD from *E. coli* and *T. thermophilus* (PDB accession codes 1D5N and 3MDS). Molecular graphics and analyses were performed with UCSF Chimera, developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco [21].

3. Results and discussion

3.1 Cloning, expression and initial purification of SodA inclusion bodies

The coding sequence for gene bb0153 was synthesized and incorporated into the MCS of pET-47b(+) for expression of *B. burgdorferi* SodA with an N-terminal 6xHis-tag. Induction of T7 Express cells with IPTG resulted in high levels of SodA expression in inclusion bodies (Fig. 1A). No detectable recombinant protein was found in the soluble fraction despite attempts to optimize expression conditions such as: induction temperature, IPTG concentration and the addition of ethanol [22] (results not shown). A series of pellet-wash steps were employed (Methods section 2.2) to isolate inclusion bodies and remove background contamination (Fig. 1B).

3.2 Metal insertion

A range of factors contributed to our experimental approach to metal insertion under denaturing conditions. Buffers containing free transition metal ions may effectively catalyze the dismutation of oxygen radicals in the absence of enzyme. In addition, because of the neutral pH and oxidizing conditions, the addition of even small concentrations of Fe^{2+} metal salts resulted in insoluble oxidation products that may interfere with subsequent colorimetric SOD assays. Furthermore, *in vitro* studies using *E. coli* Mn-SOD showed that the direct reconstitution of metal ions by dialysis is not always possible [23]. *In vivo* studies have demonstrated that the folded form of apo-SOD2 (the mitochondrial homologue of *Borrelia* SodA with 40% sequence identity) could not acquire Mn and that protein unfolding was required during mitochondrial import for metal binding and activation [24]. For these reasons, it was decided that Fe and Mn ions would be added to *Borrelia* SodA prior to refolding. The subsequent purification steps employed (Ni-NTA and size exclusion chromatography) also served to remove unbound metal ions and prevent interference with subsequent colorimetric SOD assays.

To generate denatured apo-SodA, inclusion bodies were washed in EDTA buffer, solubilized in 8M urea, and any residual metal ions were removed by passing the solution through stripped NTA resin. Metal salts (or water control) were then added to protein solutions in denaturing conditions to generate samples of apo-SodA, Fe-SodA and Mn-SodA. Centrifugation was required to remove insoluble oxidation products. All three samples (apo-SodA, Fe-SodA and Mn-SodA) were then refolded and purified independently.

3.3 Protein folding and final purification

Denatured protein samples were bound to Ni-NTA resin and allowed to refold by a slow reduction in urea concentration over 2 hours. Protein was eluted by the addition of imidazole resulting a single band on SDS-PAGE (Fig. 1C). Size exclusion chromatography was utilized to remove misfolded or aggregated protein (Fig. 1D). Three peaks were detected, the first of which corresponded with the void volume (>2000 kDa) and is most likely mis-folded/aggregated protein. The elution volumes of the two subsequent peaks correspond almost exactly to dimeric (43 kDa) and monomeric (23.5 kDa) SodA, and both peaks

were confirmed to contain SOD activity by enzyme assay. The minor peak corresponding to monomeric SodA is most likely an artifact of recombinant expression and is unlikely to be physiologically relevant. Bacterial MnSod enzymes are most frequently dimeric [25], therefore the major peak detected from size exclusion that corresponds to dimeric SodA was selected for further study. The yield of refolded proteins were similar for Mn and Fe-SodA (8 mg purified protein per liter of *E. coli* cell culture). The yield of apo-SodA was particularly low at 0.9 mg/l.

Protein folding was confirmed by circular dichroism (CD) spectroscopy (Fig. 2, Table 2). Apo-SodA, Fe-SodA and Mn-SodA were confirmed to have CD spectra with high helix content (~60%) consistent with the long alpha-hairpin N-terminal domain and the Fe, Mn SOD C-terminal domain (alpha-beta(2)-alpha-beta-alpha(2)). Values are consistent with the Mn-Sod from *Thermus thermophilus* [26], the closest homologue to *Borrelia* SodA with 51% sequence identity over 200 residues.

	α-helix	β-strand	β-turn	unordered
Apo-SodA	58 %	13 %	13 %	17 %
Fe-SodA	62 %	14 %	9 %	15 %
Mn-SodA	59 %	15 %	9 %	17 %

Table 2. Deconvolution of CD data for Apo-SodA, Fe-SodA and Mn-SodA. Proportion (%) of secondary structure types α-helix, β-strand, β-turn or unordered was determined using the CDSSTR method by DichroWeb [18, 27] using the SP175 dataset [28].

3.4 Mn activates Borrelia SodA and enhances protein folding

SOD activity was determined using an indirect colorimetric assay. Superoxide anions, produced by the oxidation of xanthine by xanthine oxidase, catalyze the reduction of WST-1 (2-(4-Iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt) to produce a formazan dye that absorbs light at 440 nm. SOD activity can be determined by measuring the inhibition of this reaction resulting from the removal of superoxide anions.

Apo-SodA and Fe-SodA failed to inhibit the enzyme activity colorimetric assay, giving average inhibition values close to zero (-1.1% ± 5.2 and 1.5% ±6.3) and were not significantly different to each other ($P>0.05$) (Fig. 3). This is in marked contrast to the Mn-SodA, which inhibited the reaction by 82.9% ±5.8. These data confirm that *Borrelia* SodA is Mn-dependent and not cambialistic.

The results demonstrate that *Borrelia* SodA was able to chelate Mn in the presence of 8 M urea, and that this Mn then remained bound to the enzyme during the subsequent refolding and purification steps resulting in active SodA. It is possible that some residual native structure exists that allows metal binding, even in the presence of 8 M urea. The refolding of apo-SodA was particularly problematic and gave a very low yield (0.9 mg/litre). Based on the improved yield of refolded protein in the presence of either Fe or Mn ions (8 mg/l), it appears that metal binding improved protein-folding. These results

are in agreement with the literature. In live *Borrelia*, levels of SodA are significantly reduced in a *bmtA* mutant unable to import Mn [15]. When *Borrelia* SodA is heterogously expressed in the mitochondria or cytoplasm of yeast cells, immunoblots revealed that protein is only detected when cells were treated with 1 mM Mn [14]. This was demonstrated to be a stabilization of polypeptide and not a transcriptional effect. Therefore, we propose that Mn-binding by SodA promotes protein folding.

3.5 Homology model of dimeric Mn-SodA

The elution volume from size exclusion chromatography indicates that SodA primarily exists as a homodimer (Fig. 1D). A homology model of the SodA dimer was generated based on the tetrameric Mn-SOD from *T. thermophilus* and the dimeric Mn-SOD from *E. coli* (Fig. 4). The primary sequence of *Borrelia* SodA contains a single Cys residue (Cys136, Fig. 4). Because of the reducing environment in the cytoplasm, most intracellular Cys residues would not form a disulfide bond, however human SOD1 has been shown to contain an intrasubunit disulfide [29], therefore the possible involvement of a disulfide in the dimer interface in *Borrelia* SodA was important to consider. The homology model of the SodA dimer revealed that the two Cys136 residues are separated by ~43 Å and are therefore unlikely to be involved in an intrasubunit disulfide without considerable rearrangement of the dimer interface. This rearrangement is decidedly unlikely as residues at the dimer interface are highly conserved between the *E. coli, T. thermophilus* and *Borrelia* proteins with only a single amino acid difference observed, Tyr34/Phe34 (Supplementary Table 1).

4 Conclusion

Borrelia SodA recombinantly expressed in *E. coli* can be refolded in the presence of Mn. This therefore allows significant amounts of protein to be produced for structural biology and biochemical studies. This Mn facilitates protein folding and remains bound to the protein throughout the subsequent purification steps. As demonstrated by indirect colorimetric assay, and consistent with known literature, *Borrelia* SodA requires Mn for activity. Finally, by studying a homology model of *Borrelia* SodA a single Cys residue at the protein surface is revealed; this may stymie future attempts at crystallography unless it is chemically blocked, effectively reduced or removed by mutagenesis. .

353 *Conflict of interest*

354 *The authors declare no conflict of interest.*

355 *Author contributions*

356 GB, DCT, AHB, & SEC performed the research, analyzed the data and helped
357 to draft the manuscript;

358 AD, AHB, GB, LS and RJB conceived and designed the work and wrote the
359 manuscript.

360 All authors contributed to and approved the final manuscript.

361

362 *Supplementary material*

363 **Supplementary Table 1 –** Residues at the dimer interface of *E. coli*, *T.*
364 *thermophilus* and *B. burgdorferi* MnSODs

Figures

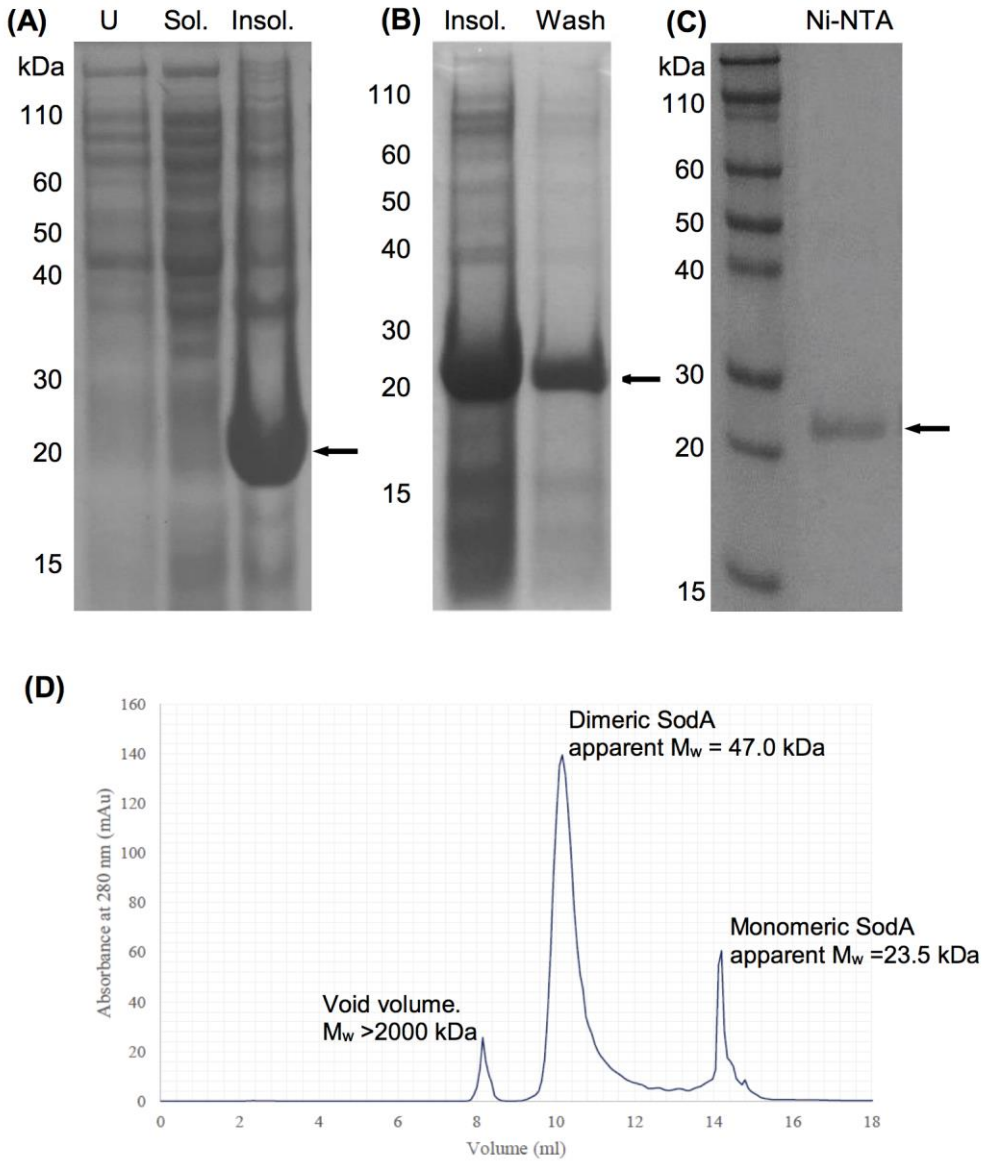


Figure 1. Purification and refolding of recombinant *Borrelia* SodA. (A) SDS-PAGE analysis of uninduced whole cell lysate (U), followed by post-induction soluble (Sol.) and insoluble (Insol.) cell lysate fractions. Recombinant *Borrelia* SodA is indicated by black arrows. (B) The insoluble pellet (Insol.) was washed six times as described in Methods (2.2) to generate a sample of inclusion bodies (Wash). (C) The inclusion bodies were then solubilized in 8 M urea and purified by immobilized metal affinity chromatography (Ni-NTA). (D) Size exclusion chromatography of *Borrelia* Mn-SodA. Fractions from both peaks were confirmed as SodA by enzyme assay.

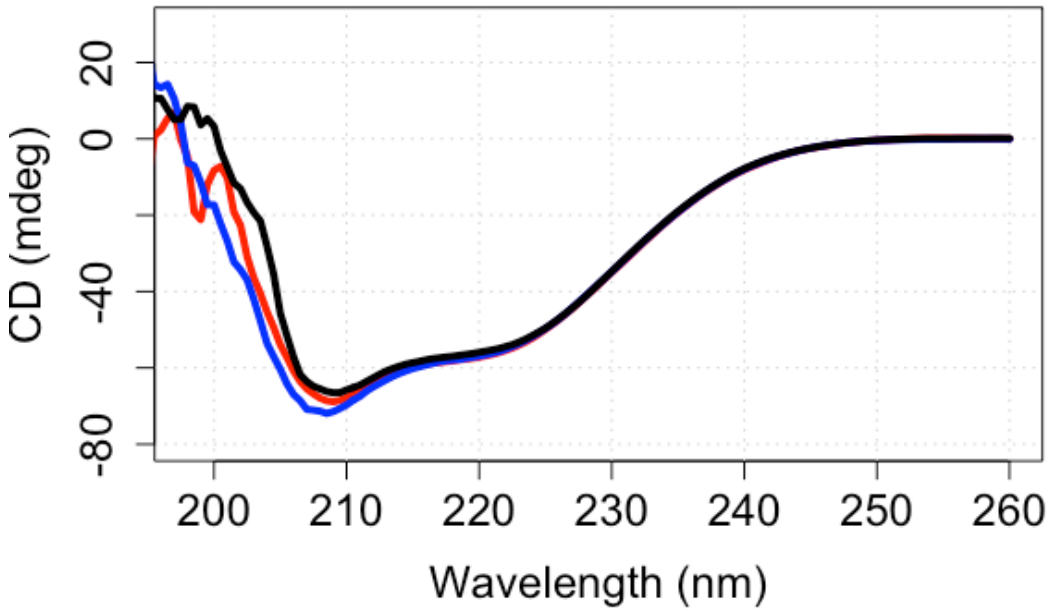
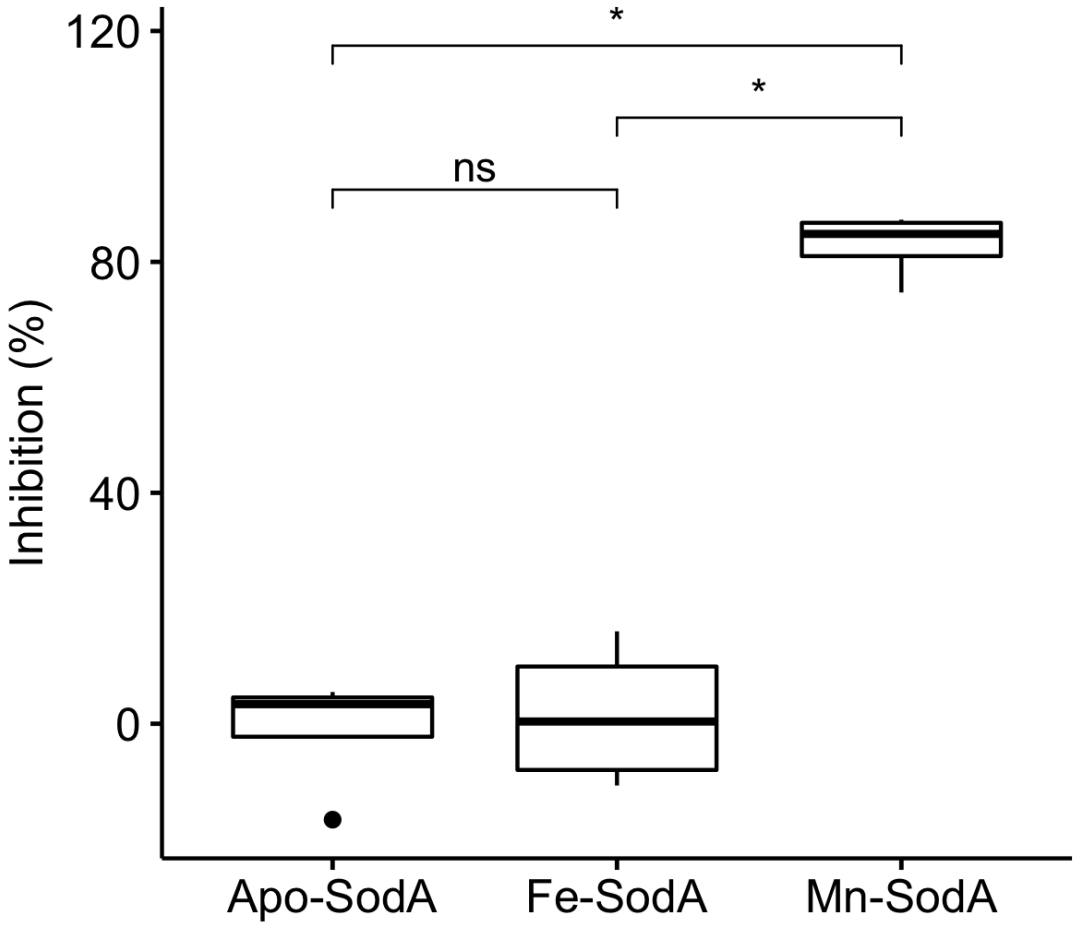


Figure 2. Circular dichroism spectra of Apo-SodA (black), Fe-SodA (red) and Mn-SodA (blue) at 20°C. Experiments were carried out on a Jasco J-810 spectropolarimeter. Experimental data was obtained over five individual scans and averaged.



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383 **Figure 3.** Activity of *B. burgdorferi* SodA refolded from 8M urea in the
384 absence of metal ions (apo-SodA), or after addition of Fe/Mn. SOD activity
385 was measured by an indirect enzyme assay based on the removal of
386 superoxide anions from solution, preventing the reduction of WST-1 to
387 formazan dye. Whiskers indicate the maximum and minimum of data, central
388 bands indicate median values. Statistical analysis by ANOVA and a multiple
389 comparison of sample means by Tukey's test revealed the enzyme activity of
390 Mn-SodA to be significantly different to both Apo and Fe-SodA ($P<0.05$).

391

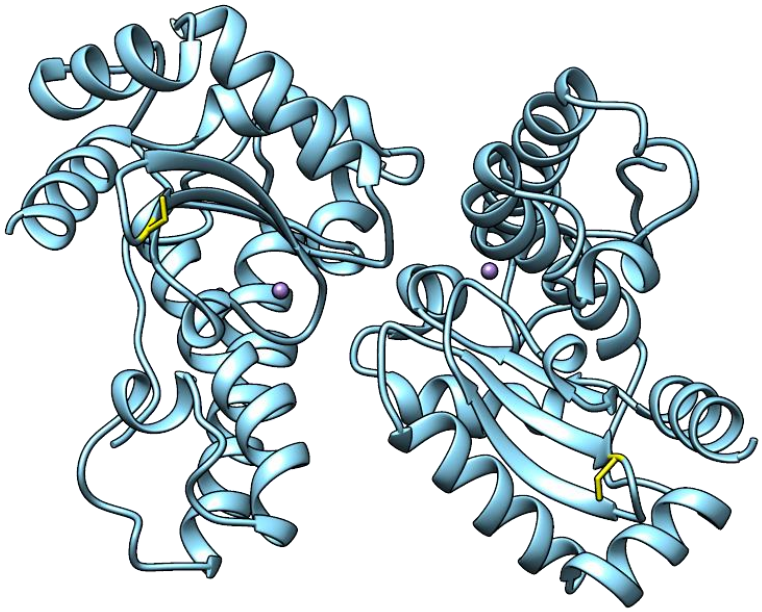

Figure 4. Homology model of dimeric *Borrelia* SodA based on *T. thermophilus* and *E. coli* Mn-SODs generated using Phyre2. 203 residues were modelled at >90% accuracy. Mn shown as purple spheres. Cys136 shown using yellow sticks representation. The distance between the two Cys residues is ~43 Å.

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